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Validation of two test systems for detecting tumor promoters and EBV inducers: comparative responses of several agents in DR-CAT Raji cells and in human granulocytes

Guy Bouvier, Manfred Hergenhahn[2], Axel Polack[1], Georg W.Bornkamm[1] and Helmut Bartsch[3]

International Agency For Research on Cancer, 150 Cours Albert Thomas, 69372 Lyon Cedex 08 and [1]GSF Forschungszentrum Hämatologikum, Marchioninistraβe 25, D-8000 München 70, Germany

[2]Visiting scientist on leave from the German Cancer Research Center, D-69 Heidelberg, Germany

[3]To whom all correspondence should be addressed

The response to a number of agents has been compared in two short-term assays used for the detection of virus inducers and tumor promoters: (i) induction of the EBV-DR-promoter in Raji cells, as measured by DR-CAT induction (DR-CAT test) and (ii) induction of the oxidative burst in human PMN, as measured by chemiluminescence in the presence of luminol or lucigenin (CL test). In order to validate the two assays, we have investigated the responses to 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1,2-dioctanoylglycerol (DAG), phospholipase C (PLC EC-3-1–4-30) and ionophore A23187, which are active in both systems; arachidonic acid, linoleic acid and NaCl were found active only in the CL test. Staurosporine (protein kinase inhibitor), tamoxifen (estrogen antagonist and protein kinase C inhibitor), forskolin (protein kinase A activator), R59949 (diacylglycerol kinase inhibitor), curcumin and *N*-acetyl-*L*-cysteine (scavengers of reactive oxygen species) and NaCl acted as inhibitors. A good concordance of the EC_{50} values of inducing substances was found between the two assays, except for A23187 and DAG, which were required at much higher concentrations in the DR-CAT test. The inhibition patterns by the panel of inhibitors revealed similarities and discrepancies in the induction pathways between the two systems, providing information on their mode of action. The two assays, which complement each other, were shown to detect a number of known or suspected EBV inducers or tumor promoters, and thus appear useful for screening of new compounds or mixtures as well as of potential antiviral and antipromoting substances.

Introduction

Epstein–Barr virus (EBV*) has been found in all biopsies from nasopharyngeal carcinoma, an epithelial tumor that is geographically mainly restricted to Southern China, North Africa and the Arctic region (1). The association between nasopharyngeal carcinoma (NPC) and EBV seems close, since EBV replication has been found to precede NPC development (2,3). In the natural history of human nasopharyngeal carcinoma, the presence of virus-inducing substances in environmental or food sources may be important. Thus, in high-incidence areas for NPC, EBV-inducing substances were found in Chinese medicinal preparations (4), plant and soil extracts (5,6) and more recently in food items (7). It is therefore important to be able to screen rapidly and quantitatively a wide variety of compounds from different sources, in order to identify EBV inducers to which humans are exposed. In these earlier studies, the induction of EBV early antigens in Raji cells by immunofluorescence detection was used as a qualitative marker for EBV induction. The use of EBV induction for the detection of potent tumor promoters was proposed in 1984 by Takada and zur Hausen (8), who tested some suspected human tumor promoters. The DR-chloramphenicol acetyltransferase (CAT) system described by Polack *et al.* (9) and used here, is a further step towards developing simple and quantitative screening tests for EBV-inducing activity.

Some interactions of EBV and chemical carcinogens have been previously investigated (for a review see 10), and several other compounds were also found to exert *in vitro* EBV-inducing activities (see 9). A number of EBV inducers are also tumor promoters in animal models, and some are suspected human tumor promoters, e.g. butyrate (11). Interestingly, combinations of some of these inducers [e.g. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and butyric acid] have shown an over-additive effect, whereas in other cases [e.g. TPA and teleocidin, two tumor promoters which activate protein kinase C (PKC)], only additive effects were observed (12,13); these data suggest that different induction pathways for EBV exist, a major one of which is PKC activation (14,15). Furthermore, AP1-responsive elements which are involved in EBV replication have been identified as the TPA-responsive elements (TRE) that are activated indirectly by PKC (27, 34).

In order to validate more quantitative and rapid test systems for EBV inducers and tumor promoters acting through different pathways, we have investigated a series of reference compounds in two systems side by side: (i) in the DR-CAT test and (ii) in the granulocyte–chemiluminescence (CL) test. The former uses Raji cells stably transfected with an autoreplicative plasmid carrying the EBV-DR promoter that controls the CAT reporter gene (9); EBV inducers are quantified by measuring the conversion of [^{14}C]chloramphenicol to mono- and di-acetylated derivatives. This provides a rapid and quantitative measure of the potency of an EBV inducer. The present study is the first to validate the DR-CAT-Raji system by determining concentration-response relationships of different classes of inducers, and of some response modifiers (inhibitors); in addition, a parallel study on human polymorphonuclear cells (PMN) CL comparing the luminol- and lucigenin responses to this group of inducers was to our knowledge not described before. In the CL test, PMN are induced by various compounds to produce an oxygen burst by activation of NADPH-oxidase through several pathways, a major one again involves PKC (16,17).

*Abbreviations: EBV, Epstein–Barr virus; NPC, nasopharyngeal carcinoma; CAT, chloramphenicol acetyltransferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; TRE, TPA-responsive elements; CL, chemiluminescence; PMN, human polymorphonuclear cells; DAG, 1,2-dioctanoylglycerol; PLC, phospholipase C; PBSGA, phosphate-buffered saline, containing 0.1% glucose/bovine serum albumin (w/v); NAC, *N*-acetyl-*L*-cysteine; Cl-Lm, CL in the presence of luminol; CL-Lc, CL in the presence of lucigenin; EC_{50}, effective concentration 50%; IC_{50}, inhibitory concentration 50%; PKA, protein kinase A; CA, chloramphenicol.

The aims of this study were (i) to investigate a number of virus inducers, tumor promoters and inhibitors for their ability to induce or inhibit DR-CAT expression in Raji cells, and NADPH-oxidase in PMN, in order to find out whether the two biological responses are induced by similar pathways, (ii) to validate a test system for detecting EBV inducers based on CL measurement in PMN and (iii) to assess the role of the PKC pathway in EBV activation by using inhibitors.

Materials and methods

Chemicals

TPA, arachidonic and linoleic acid, dioctanoylglycerol (DAG), tamoxifen, staurosporine, forskolin, phospholipase C (PLC) type XIV from *C. perfringens* (EC-3-1−4-30), bovine serum albumin, phosphoenolpyruvate and *N*-acetyl-L-cysteine were from Sigma (L'Isle d'Abeau, France). Ionophore A23187, hygromycin B, chloramphenicol and acetyl-CoA, luminol and lucigenin were obtained from Boehringer (Meylan, France). R59949 was from Janssen (Paris, France). Solvents of analytical grade, glucose, curcumin, sodium chloride and butyric acid were from Merck (Nogent sur Marne, France). All stock solutions were kept at high concentrations at −20°C.

RPMI 1640 was purchased from Vietech (St Bonnet de Mure, France); antibiotics from Flow (Paris, France), fetal calf serum from PAA (Vienna, Austria), [^{14}C]chloramphenicol from NEN Dupont (Paris, France) and dextran 200 000 from Fluka (Altkirch, France).

Cell culture and CAT assay

DR-CAT Raji is a human B lymphoma cell line derived from a Burkitt's lymphoma, stably transfected with an autoreplicative plasmid containing the chloramphenicol acetyltransferase gene under the control of the EBV DR early promoter (9). Cells were grown in RPMI 1640, 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin, 300 μg/ml hygromycin B at 37°C, in a humidified atmosphere with 5% CO_2. Cells were used in the logarithmic growth phase (>90% viable cells/assay); 3 ml of a cell suspension at 2×10^5 cells/ml was incubated with test compounds for three days, along with solvent controls. For inhibition experiments, the inhibitor was preincubated for 90 min with cells before addition of the inducer. In the case of the ionophore A23187, 0.5 mM $CaCl_2$ was added to the culture medium. As positive controls cells were incubated with TPA at either 32 nM (20 ng/ml, maximal induction) or 0.75 nM (half-maximal induction). After exposure, cells were harvested, washed with cold PBS and lysed by sonication in 300 μl PBS (twice for 5 s; Branson sonicator). Cell extracts were obtained after centrifugation for 10 min at 10 000 r.p.m. at 4°C, and then incubated at 60°C for 10 min (18). Protein concentrations of the cell extracts were determined by the Bradford method; CAT assays were done with 25 μg protein/assay for 30 min as described by Gorman *et al.* (19). After autoradiography, radioactivity in zones of the TLC plate was quantified by LSC, and the yield of acetylated chloramphenicols was expressed as the percentage of the total radioactivity per assay. Effective concentration 50% (EC_{50}) is the concentration giving 50% of the maximal conversion of chloramphenicol observed for each inducer, determined graphically by interpolation.

Isolation of neutrophils from blood

Blood (20 ml) from healthy donors (Centre Régional de Transfusion Sanguine, Lyon, France) was separated by sedimentation of erythrocytes with PBS/2.5% dextran (mol. wt. 200 000) for 30 min at room temperature. Cells in the erythrocyte supernatant were spun down for 5 min at 900 *g*. Residual erythrocytes were lysed by hypotonic shock with 0.2% NaCl for 45 s. Cells were washed with cold PBS/0.1% glucose/0.1% albumin (w/v) (PBSGA), resuspended in 5−10 ml of the same buffer and kept on ice. According to Coulter counter measurement, the cell population consisted of ~10% of cells >7 μM; it is only these large cells that were counted as PMN. This separation procedure was used routinely after we noted that the conventional method of white cell separation on Ficoll resulted in activated cells which gave only a lowered oxygen burst after TPA treatment.

CL measurements

CL measurements (CL test) were performed using a LKB-1251 luminometer; ~10 μl of the leukocyte cell suspension (~50 000 cells) were incubated in PBSGA with either luminol or lucigenin at a final concentration of 0.1 mM (final vol 1 ml). Luminol is known to act both extra- and intra-cellularly and to recognize mainly hypochlorite or a related oxidant (20); lucigenin detects mainly superoxide anions and hydrogen peroxide extracellularly (21). In order to standardize the method and to correct for differences between blood donors, the number of cells (~50 000) was adjusted so as to give an instrument response of ~200 mV in the presence of 100 nM TPA (the concentration giving the maximal peak height). In experiments with the ionophore A23187, 0.5 mM $CaCl_2$ was added to the incubation buffer. CL was measured in mV/s until the signal returned to background levels. The integrated response (mV × time) was calculated and the integrated background signal was subtracted. Solvent controls were performed with and without the inducers. Inhibitors were incubated for 5 min with cells in cold assay buffer before addition of the test substance. CL measurements were performed at 37°C immediately after vortexing of the tube and transfer into the LKB luminometer. The EC_{50} was obtained as the concentration giving an integrated signal corresponding to 50% of the maximal response for each inducer, determined graphically by interpolation.

Pilot experiments were performed to determine the toxicity of each compound, and the maximal concentrations were chosen so that at least 70% of the cells were viable at the end of incubation.

When inhibition studies were performed, we determined graphically the concentration inhibiting 50% of the activity [inhibitory concentration 50% (IC_{50}) value] of the inducers tested at their EC_{50} in the case of the DR-CAT test, and reducing the integrated signal by 50% in the case of the CL test. The percentage of inhibition or stimulation was calculated for each concentration of a given inhibitor in both tests as: 100× (response with the inhibitor−background response)/(response with EC_{50} of the inducer−background response).

Results

EBV induction as revealed by CAT activity and determination of EC_{50} values for some EBV inducers

EBV-DR-CAT induction in Raji cells was tested with TPA, DAG, PLC from *C. perfringens* (EC 3-1−4-30), the Ca^{2+}-ionophore A23187, arachidonic and linoleic acids; the concentration ranges tested, the maximal responses obtained and the EC_{50} values are given in Table I.

EBV-positive Raji cells containing the DR-CAT plasmid were treated with TPA at concentrations from 0.1 nM to 10 μM. The highest concentration was found to be less active, the maximal response (75.6% conversion of [^{14}C]chloramphenicol) was observed at 100 nM; the dose-response curve showed an EC_{50} of 0.75 nM (see Figure 1). DAG was tested between 500 nM and 1 mM. The maximal response was found at 1 mM with only 5.9% conversion ± 5.1 (see Figure 1). The maximal response to PLC was detected at 2 U/ml (18% ± 4.4) with the EC_{50} at 0.5 U/ml (see Figure 1). Ionophore A23187 showed its highest activity between 250 and 500 nM; at 10 nM, no induction was detected. However, the range for CAT induction was extremely wide (250 nM gave 35.2% ± 35). Thus, the EC_{50} was fixed at 250 nM for further experiments. Arachidonic and linoleic acids tested between 1 nM to 1 mM gave no induction of the DR promoter (results not shown). It was noted that as the concentration of TPA increased, the level of induction began to decrease before toxicity became very important; such a bimodal effect was not seen with A23187, PLC or DAG.

Determination of the EC_{50} values of some inducers in the CL test using luminol and lucigenin

As shown in Table I, induction of CL by the diterpene ester TPA was observed with the two luminophores in the concentration range 0.1 nM to 10 μM showing an early peak (at ~6 min). The EC_{50} was determined with both luminophores to be 1 nM. Concentrations below 0.1 nM TPA still gave a slight response with both luminophores. The maximal values obtained (for the same number of cells/assay) were ~200 mV at 100 nM TPA with luminol, but only ~11 mV with lucigenin under the same conditions.

DAG activity was hardly detectable below 5 μM. As with TPA, the responses observed with the two luminophores were different (compare Figure 2A and C), although the EC_{50} values were the same (7 μM). This EC_{50} value is 7000-fold higher than that observed for TPA.

PLC type XIV from *C. perfringens* was tested in the CL test with both luminophores, but a significant response was found

Table I. EC_{50}, range of active concentrations and maximal responses observed (in terms of integrated signal) for the different inducers in both tests

Inducer	Luminol	Lucigenin	DR-CAT induction
TPA			
EC_{50}	1 nM	1 nM	0.75 nM
Concentrations	0.1 nM:10 μM	0.1 μM:10 μM	0.1 μM:10 μM
Max. response	200 mV (100 nM)	11 mV (100 nM)	76% (100 nM)
A23187			
EC_{50}	50 nM	50 nM	250 nM
Concentrations	10 nM:10 μM	10 nM:10 μM	50 nM:0.5 μM
Max. response	160 mV (100 nM)	6 mV (2.5 μM)	37% (500 nM)
DAG			
EC_{50}	7 μM	7 μM	n.d.
Concentrations	1:250 μM	1:250 μM	10 μM:1 mM
Max. response	140 mV (80 μM)	7 mV (20 μM)	5.1% (0.5 μM)
PLC			
EC_{50}	0.6 U/ml	0	0.5 U/ml
Concentrations	0.1:10 U/ml	0	10 mU:2 U/ml
Max. response	35 mV (10 U/ml)	0	18% (2 U/ml)
Arachidonic acid			
EC_{50}	0.75 mM	0.5 mM	0
Concentrations	0.25 mM:1 mM	0.25 mM:1 mM	0
Max. response	600 mV (1 mM)	134 mV (750 μM)	0
NaCl			
EC_{50}	0.246 M	0	0
Concentrations	0.181:0.483 M	0	0
Max. response	0.395 (8 mV)	0	0

n.d., not determined.

only with luminol. 10 U/ml gave the highest response in terms of peak height at ~4 min (before the peak induced by any concentration of TPA or DAG); the EC_{50} was ~0.6 U/ml.

Ca^{2+}-ionophore A23187 activities were ~2–4 times higher in the presence of calcium at 500 μM (results not shown). Two subgroups of blood donors were identified in terms of the response of their PMN cells to A23187 in the CL-test. Cells from the major subgroup ('high responders') were used for the following experiments with A23187, and gave a rapid and transient peak above 1 μM; the maximal peak value was at 100 nM with a level ~60% of the TPA peak at 100 nM (see Figure 2A). The activity of A23187 decreased steeply below 50 nM to a level just above background at 10 nM (see Figure 2B); this prevented accurate determination of its EC_{50} by graphical means, but the value was estimated as 50 nM.

Arachidonic acid at 1 mM gave an extremely high but very transient response, with the peak level five times the TPA response, in ~1 min (see Figure 2A). (The response decreases rapidly at lower concentrations, with low response levels at 0.25 mM). Similar results were obtained with both luminophores.

Different NaCl concentrations were used in PBSGA, and CL was observed only in the presence of luminol (see Figure 2B); the EC_{50} was 0.246 M, taking into account the NaCl concentration in PBSGA (see Table I). Preincubation of cells with NaCl or addition of NaCl concomitantly with TPA, inhibited the TPA response (not shown).

Response modifiers in the DR-CAT test and the CL test

Six inhibitors were used to probe the induction pathway(s) in the two test systems: staurosporine, a strong inhibitor of several protein kinases (22); tamoxifen, an estrogen antagonist and a specific inhibitor for PKC that interacts with membrane lipids (23,24); forskolin, an activator of protein kinase A (25); R59949, a specific inhibitor of diacylglycerol kinase (26); curcumin, an inhibitor of AP1 action (27) and an antioxidant (28,29); *N*-acetyl-*L*-cysteine (NAC) as a precursor of glutathione; and NaCl, known to activate the Na^+/K^+ pump in PMN (30).

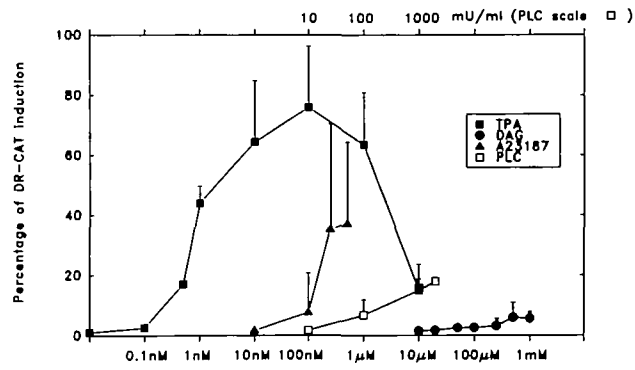

Fig. 1. Induction of EBV activation by TPA, DAG, PLC and A23187, as revealed by the DR-CAT test, expressed in % CA conversion. Means ± SD are plotted from at least three independent experiments run in duplicate.

For CL experiments, inhibitors were tested with both luminol and lucigenin with TPA at its EC_{50} = 1 nM, DAG EC_{50} = 7 μM, PLC EC_{50} = 0.6 U/ml, A23187 EC_{50} = 50 nM and NaCl (used at its maximally active concentration) 0.395 M. In the EBV-DR-CAT test, inducers were tested at the following EC_{50}: TPA 1 nM, PLC 0.5 U/ml, A23187 250 nM and compared with controls containing 0.17% DMSO (5 μl added to 3 ml assay), since we observed that this concentration of DMSO alone reduced the induction of EBV-DR-promoter by TPA (0.75 nM) by ~40%. Due to the very high EC_{50} value determined for DAG in EBV induction, inhibition experiments were done for this compound in the DR-CAT test only with R59949.

As described in Figure 3A, staurosporine was inhibitory for all inducers in the two assay systems, with a range of IC_{50} from 0.5 to 2.2 nM, except for A23187 where the IC_{50} values were

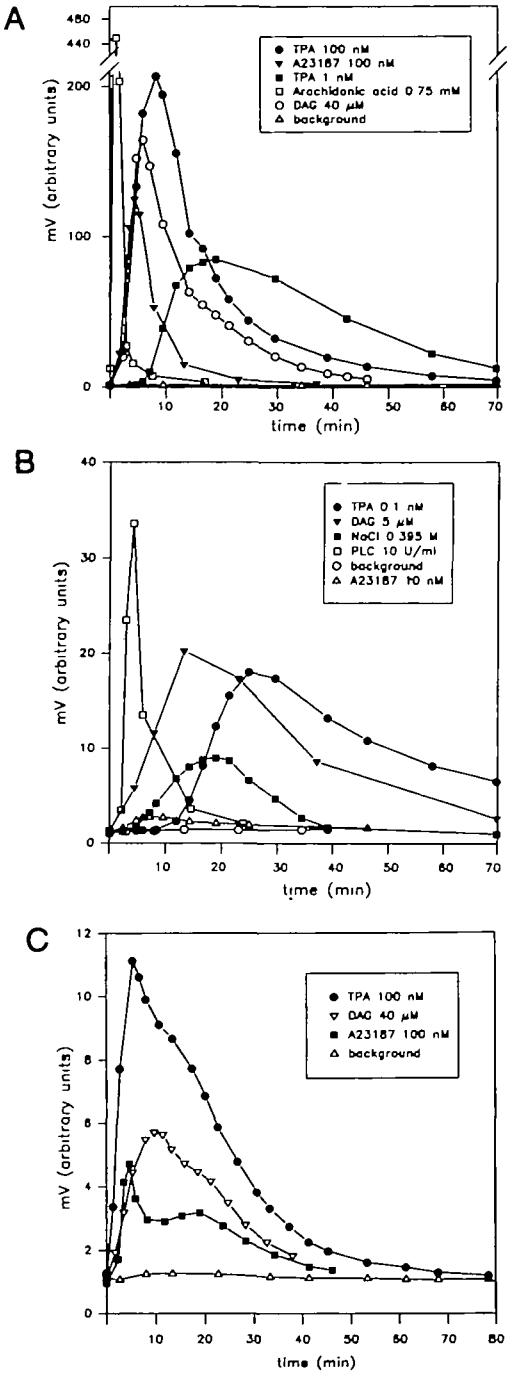

Fig. 2. (**A**) CL response in presence of luminol with TPA, A23187, DAG and arachidonic acid, presented at their maximal active concentrations, and comparison of the curve obtained with TPA at 1 nM. (**B**) CL response in presence of luminol with TPA, A23187, DAG at their minimally detectable concentrations for comparisons with PLC and NaCl at their maximal active concentrations. (**C**) CL response in presence of lucigenin with TPA, A23187 and DAG at their maximal active concentrations.

determined at 100 nM for EBV-induction, and 21.8 and 4.4 nM for CL-induction in presence of Lm and Lc respectively. Tamoxifen was not found to modulate TPA action on EBV induction, whereas it increased EBV induction by PLC and inhibited the A23187 effect. CL-induction by all compounds was inhibited in the range of concentrations from 2.1 to 5.8 µM (see Figure 3B). Forskolin action (described in Figure 3C) led to inhibition of EBV induction by TPA, A23187 and PLC at 10, 2.2 and 0.22 µM respectively. Neither CL in the presence of

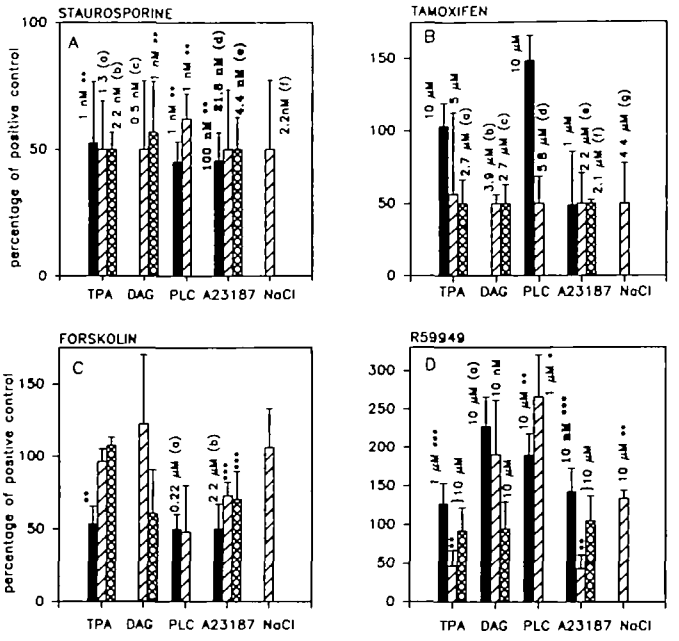

Fig. 3. Inhibition by various inhibitors (indicated below) of TPA, DAG, PLC, A23187 and NaCl action tested at their EC_{50} in the DR-CAT-test (■), in the CL-test with Lm (▨) and Lc (▩). Means ± SD are calculated from at least three independent experiments run in duplicate if not otherwise specified. Statistical significance for inhibition (or increase) of response as compared to control assays (100%) is given as : * $P < 0.001$; ** $P < 0.01$ and *** $P < 0.05$. IC_{50} are indicated on top of each bar (either determined directly or by graphical interpolation). (**A**) Staurosporine. a) value interpolated between 1 nM (62.5% ± 21.1) *** and 2.5 nM (21.6% ± 11.9) **; b) 1 nM (67% ± 11.2) *** and 5 nM (32.4% ± 2.2) **; c) 0.1 nM (110.4% ± 46.9) and 1 nM (22.8% ± 7.1) **; d) 10 nM (75.2% ± 39.6) and 100 nM (4.6% ± 7.2) **; e) 1 nM (141.4% ± 24.9) and 10 nM (0%) *; f) 1 nM (97.7% ± 26.9) and 10 nM (38.1% ± 28.4) **; (**B**) Tamoxifen a) 1 µM (80.8% ± 12.3) ** and 10 µM (22.7% ± 21.3) **; b) 1 µM (100.4% ± 9.4) and 10 µm (16.5% ± 2.6) **; c) 1 µM (81.86% ± 4.11) *** and 10 µM (7.14% ± 4.5); d) 10 µM (41.2% ± 17.2) *** and 1 µM (81.4% ± 20.1); e) 1 µM (75.8% ± 31.1) and 10 µM (4.8% ± 10.4) **; f) 1 µM (70.8% ± 2.5) ** and 10 µM (0%)*; g) 1 µM (89.9% ± 36.6) and 10 µM (21.5% ± 18.6) **; (**C**) Forskolin was used at 10 µM if not otherwise indicated. a) 100 nM (69.4% ± 10) *** and 1 µM (25.1% ± 9.2) **; b) 1 µM (69.2% ± 22) and 10 µM (16.6% with a range of 10 and 23.2% in 2 experiments); (**D**) R59949 a) range from two experiments is given, using only the high concentration of DAG (500 µM).

luminol (CL-Lm) nor CL in the presence of lucigenin (CL-Lc) induction by any of the compounds tested appeared significantly modified. R59949 increased EBV-induction by any of the inducers (see Figure 3D), the most effective concentrations were 10 µM for PLC and DAG, 1 µM for TPA and 10 nM for A23187. In contrast, CL-Lm induction by TPA and A23187 was inhibited at 10 µM R59949, stimulation of the responses of DAG, PLC and NaCl was observed for R59949 concentrations of 10 nM ($P < 0.05$), 1 µM ($P < 0.001$) and 10 µM ($P < 0.01$) respectively. No significant modification was observed in the case of CL-Lc.

Curcumin (two experiments run in duplicate) gave an IC_{50} of ~10 µM in the DR-CAT test (see Figure 4), ~1 µM in the CL test with luminol and 5 µM with lucigenin. The EC_{50} of the glutathione precursor *N*-acetyl-*L*-cysteine (NAC) was ~5 mM (see Figure 4) in the DR-CAT test. NAC also decreased the TPA response in the CL test, but it was observed that a neutralized NAC solution alone already induced a PMN response, similar to the situation observed with NaCl (results not shown).

NaCl inhibited TPA induction of EBV with an IC_{50} of 125

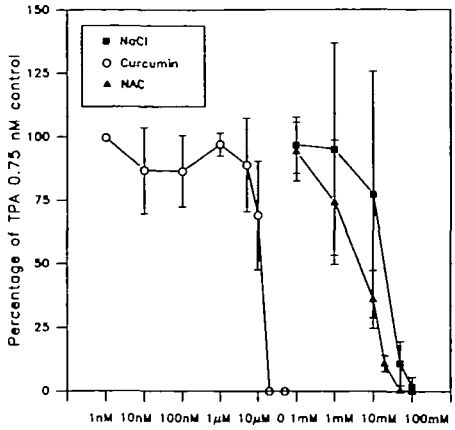

Fig. 4. Inhibition by curcumin, NaCl (without correction for the NaCl already present in the medium) and *N*-acetyl-*L*-cysteine of EBV induction by TPA (0.75 nM) in the DR-CAT test. When corrected for the NaCl already present (0.103 M) in the medium, and the volume of the NaCl solution added, the concentrations of NaCl were: 100.8, 101.6, 110.5, 151.1 and 200.3 mM.

mM (taking into account the NaCl present in RPMI) (Figure 4). In PMN, NaCl at 0.395 M induced some CL, but simultaneous or subsequent treatment of the cells with TPA did not give the typical TPA response; this response was also not restored with phosphoenolpyruvate at 100 mM which should replete the ATP pool (30)(results not shown).

Discussion

To study both EBV induction in the Raji DR-CAT system and oxidative burst generation in PMN, we used TPA, DAG, A23187 and PLC; the comparison of the EC_{50} values determined showed a good correlation between the CL test in presence of luminol and DR-CAT induction (see Table I). An exception to the correlation of EC_{50} values is DAG for which the EC_{50} value is ~7000 higher than that of TPA in the CL test. Though some induction by DAG was seen in the DR-CAT test, a meaningful EC_{50} could not be established. The EC_{50} for TPA observed in both systems is close to the K_d of PKC for TPA; while a 7000-fold higher EC_{50} for DAG in the CL test is comparable to that observed in another system (31), the low level of CAT induction seems to indicate that DAG action is very tightly controlled in DR-CAT Raji cells.

Inhibition of induction

The IC_{50} values for each inhibitor tested indicate that staurosporine, a general protein kinase inhibitor, gives similar inhibitions of both EBV- and CL-induction (Figure 3A), except for A23187 which is inhibited with staurosporine concentrations 100-fold higher than that observed with the other inducers in the case of EBV, indicating probably an induction mechanism other than through protein kinases. Surprisingly, tamoxifen, a specific inhibitor of PKC in certain cell systems, did not inhibit DR-CAT induction by TPA and even enhanced the PLC response whereas it inhibited all compounds in CL induction; it may be assumed that this effect is related to changes in the lipid phase of the Raji cell membranes. Forskolin inhibited the induction of EBV by TPA, A23187 and PLC (at the lowest forskolin concentrations); this suggests an involvement of protein kinase A (PKA) in the regulation of induction of EBV *in vitro* (also observed in the case of induction of EBV in AKATA cells by IgG crosslinking; 32), whereas it seems that the oxygen burst in PMN monitored by luminol is independent of PKA. Significant increases for exogenous phosphatidylcholine-specific PLC and DAG responses with R59949 in the CL test confirm the effect of this inhibitor on DAG turnover. R59949 has been found to double the low level of induction of EBV by DAG. This suggests either a higher level of DAG metabolism through other pathways than DAG-kinase, or a decoupling of exogenous DAG action from the PKC-EBV inducing pathways in the DR-Raji cell line used, which could account for the tight latency of this cell line (14). Surprisingly, induction of EBV by TPA was also increased by R59949, suggesting some phospholipid cycle activation by TPA leading to DAG formation (33). PLC activates EBV in Raji cells and the oxygen burst in PMN where its effect is strongly enhanced by R59949 and appears even before the effects of TPA or exogenous DAG. This suggests that DAG generated externally by a specific PLC is much more efficient in activating subtypes of PKC linked to EBV induction and oxidative burst. The action of R59949 on the induction of CL in presence of luminol by NaCl, suggests that high NaCl concentrations may indirectly activate lipid cycles.

As shown by Davies *et al.* (14), H-7 (a relatively specific inhibitor of PKC) and a synthetic pseudopeptide PKC (19–36) do inhibit PKC-dependent induction of viral genes in EBV-transformed B-lymphoid cell lines, underlining the importance of the PKC pathway. Our present data in the new system confirm these results. In addition, they show that Ca^{2+} uses another mechanism which induces maximally 50% of the CAT level as induced by TPA. It has previously been demonstrated that PKC activation by TPA leads to DNA binding of transcriptional regulators to AP1 sites (34); this interaction is inhibited by curcumin (27). We found inhibition of EBV induction by curcumin and its inhibition of the oxidative burst in PMN after TPA treatment, the latter in accordance with previous data in the same cell type (29). This may be related to the alleged antioxidant (35,36) and antipromoter (37,28) effects of curcumin. In this context, it is interesting that curcumin is commonly used in traditional medicine in India as an antineoplastic and antiviral agent (J.Nair, personal communication).

In conclusion, this study has validated the use of DR-CAT induction and the CL tests in screening potent EBV inducers from different sources. Application of both tests can provide information on the mechanisms of action of compounds tested, based on their kinetics in the CL-test and their inhibition patterns in both tests.

Secondly, this study and previous work on EBV-inducing activities (38,8,15,9) and similar data on induction of CL in PMN (39,40,17) show that a wide range of potent and potential tumor promoters can be detected by at least one of these tests. For example, sodium butyrate does not induce a PMN response but induces *in vitro* replication of EBV (41) and DR-CAT induction (9), which can be inhibited by staurosporine and forskolin but not by tamoxifen and R59949 (Bouvier and Hergenhahn, unpublished results). NaCl, which when consumed excessively is associated with gastric cancer risk, gives a positive response in the CL test, and its action is also modulated by some inhibitors. However, PMN (and Raji cells) in presence of elevated concentrations of NaCl do not respond to TPA, and possibly also not to endogenous activators, e.g. bacteria.

Finally, our study indicates the potential of the PMN test to investigate response differences between PMNs from individual humans, to a panel of inducers. Whether these differences are related to individual sensitivity to toxic environmental agents remains to be investigated. In a subsequent paper, we shall describe the use of these test systems for isolation of strongly

EBV-inducing fractions from a food source (Hergenhahn *et al.*, in preparation), whose consumption is associated with an increased risk for NPC.

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